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Effect of strenuous physical activity and *Crocus Sativus* supplementation on inflammatory biomarkers in male mountain climbers

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A B S T R A C T

Background: This study attempted to investigate acute effect of strenuous physical activity with and without *Crocus Sativus* supplementation on heat shock protein 70 (HSP70), interleukin-6 (IL-6) and TNFα in male mountain climbers.
Methods: A 10-day, single-blinded semi experimental design was adopted. Eleven male climbers with an age range of 27-37 years old ran at one session strenuous physical activity with intensity 80-90% of maximum heart rate for 45 minutes on a -5% slope surface. Blood samples were collected from subjects before, immediately and one hour after the strenuous physical activity to measure HSP70, IL-6 and TNFα. 3 days after recovery, the subjects were given 300 mg of dried stigmas of *Crocus Sativus* in capsules once a day for ten days. Subsequently, blood samples were obtained from the subjects again before, immediately and one hour after strenuous physical activity. The changing trends in the variables were evaluated through analysis of variance with repeated measures at a significance level 0.05.
Results: One session strenuous physical activity with *Crocus Sativus* supplementation compared with only strenuous physical activity had a significant effect on the level of heat shock protein 70 680.25±127.86 (ng/ml) vs 528.18±111.63 (ng/ml) at the immediately after of strenuous physical activity ($p<0.05$).strenuous physical activity with *Crocus Sativus* supplementation compared with only strenuous physical activity had no significant effect on the level of IL-6 and TNFα at the before, immediately and one hour after strenuous physical activity ($p>0.05$).
Conclusion: *Crocus Sativus* intake ten days before a session of strenuous physical activity can decrease the effect of heat shock protein 70 immediately after strenuous physical activity.

Introduction

One session strenuous physical activity may alter the immune system. It has been demonstrated that after the activity, there is decreased immune function for a period of time. This may create an open window in the immune system, leaving the body prone to invasion of viruses and bacteria, thus increasing the risk of infection [1, 2].

Strenuous physical activity can be extremely stressful, challenging the homeostasis of the body, to the extent that the athlete needs to restore the balance of new dynamics in order to minimize the damage to the cells [3].The body's

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response to exercise-induced stress is a widespread systemic effort of coordination by all organs, including the immune system [4, 5].

Organisms are protected against many damaging effects of stress through different strategies, in one of which the cells can confront damage or stress-induced cell death [6]. Synthesis involves a set of proteins known as HSPs. Some studies have found that exercise may be a potent stimulus for the emergence of heat shock protein [7].

Skeletal muscle in response to exercise is the main source of cytokines.

Cytokines released from skeletal muscle not only are related to exercise-induced immune changes, but also are mediators of acute exercise-induced metabolic changes and adaptations to exercise.

Increased Serum IL-6 depends on the duration and intensity of activity, muscle mass and endurance capacity of the individual involved. Serum IL-6 is more sensitive to the intensity of exercise[8, 9].

TNFα is a primary mediator of local inflammatory , also it acts as the initiator (starter) of acute phase response [10]. The general effect of IL-6 and TNF-α Results in production of acute phase proteins and fever [11].

Circulating levels of inflammatory mediators are often strongly correlated with each other as a result of their tight, regulated production. So that strenuous physical activity increased the il6 this increase is followed by the appearance of IL-1 receptor antagonist (IL-1ra), the anti-inflammatory cytokine IL-10 and sTNFRs. Increased IL-1ra IL-10 and sTNFRs leading to inhibition of TNF_{α} [8, 9, 11].In general, the difference in cytokine response to exercise and infection, according to TNFα, so cytokine response to exercise is not associated with increased TNFα.

Ziemann et al. (2013) examined the effects of exercise on inflammatory mediators and heat shock protein among young male tennis players. In this study, HSP70, IL-6, and TNFα were evaluated after the season (arriving at the training camp), after 3 days of active resting and after 10 days of strenuous training. The pro-inflammatory cytokines such as TNFα and HSP70 reduced while anti-inflammatory cytokines such as IL-6 increased [12].

Riemann et al 2013 examined the effects of IL-6, TNF-α, in response to exercise duration. In this study 22 Male Half Marathon Runners, who regularly exercised with moderate intensity and 18 male marathon runners with regular high intensity exercises was attended. Changes were measured two days before, 15 minutes and 48 hours after exercise. IL-6 and TNFα increased after exercise, but this increase was higher in marathon runners[13].

Recently, adverse effects of some industrial supplements have shifted the focus of sports science researchers to the intake of herbal supplements. Technically known as Crocus sativus, saffron crocus is the common name of a plant of the species of Iridaceae, one of the herbs that have numerous medicinal and therapeutic properties. *Crocus Sativus* stigma contains more than 150 chemicals among which crocin, picrocrocin, safranal play the key roles in producing the pharmacological effects of *Crocus Sativus* [12].

The influential factors in reducing the level of athletic performance are fatigue, decreased immune function, followed by disease [14, 15]. Given the pharmacological effects of *Crocus Sativus* (antioxidant, anti-cancer, improved atherosclerosis and the related diseases, high blood fat and cholesterol, hyperinsulinemia, high blood pressure, and insulin resistance[16, 17]. The present study was designed and conducted to find an answer to question how a session strenuous physical activity with and without *Crocus Sativus* supplementation can affect the levels of HSP70, IL-6 and TNFα in male mountain climbers.

Materials and Methods

This was a semi-experimental study, where the research population involved Tehranian male climbers aged 27 to 37, with minimum 2 years mountaineering and climbing experience at minimum height of 4000 meter and a minimum height of 3500 m bivouac, who were not smoking, overweight and no history of endocrine diseases, diabetes, heart disease and chronic disease. Among the 27 to 37 year-old male mountaineers in Tehran volunteering to participate in the study, 11 climbers were selected through targeted sampling based on a questionnaire completed about general health and level of strenuous physical activity.

Table 1. The training protocol

Phase	Speed (Kilometers per hour)	Time (min)	Slope
Warm up	5	0-5	0
Running	8	5-10	-5
Running	8.5	10-15	-5
Running	9	15-20	-5
Running	9.5	20-25	-5
Running	10	25-30	-5
Running	10.5	30-35	-5
Running	11	35-40	-5
Running	11.5	40-45	-5
Running	12	45-50	-5
Cool down	8	50-55	0

Participants were selected from male mountaineers in Tehran with an average age of 31.5±3.89 (30 mountaineers). After initial physiological data collection, and performing Body Analysis Tests 13 mountaineers were excluded and 17 remained to participate in the study. 6 persons were excluded because of not participating in the second exercise session and lack of saffron supplements and finally, 11 persons were completed the study.

Having been briefed about the conditions and completed the written consent forms, the subjects systematically participated in the research project. All subjects were evaluated and matched in terms of the general state of health and wellness, health records and diseases, medications, current diet and daily strenuous physical activity. Considering that age and gender are among the factors contributing to the research variables, both were selected homogeneously.

Training protocol

At the pre-test stage, the subjects were asked to maintain their normal sleep patterns (at least 8 hours a night), daily activities and diet patterns (12 hours of fasting before testing). In addition, all subjects were advised to avoid strenuous physical activity particularly strenuous physical activity and climbing to altitudes above 2000 m above sea level up to 72 hours before participation in the training program (Table 1).

Food frequency of almost all subjects in terms of calories and fat intake and carbohydrates and protein intake were similar. Daily intake of macronutrients including carbohydrates (55%), protein (15%) and fat (30%) were recommended

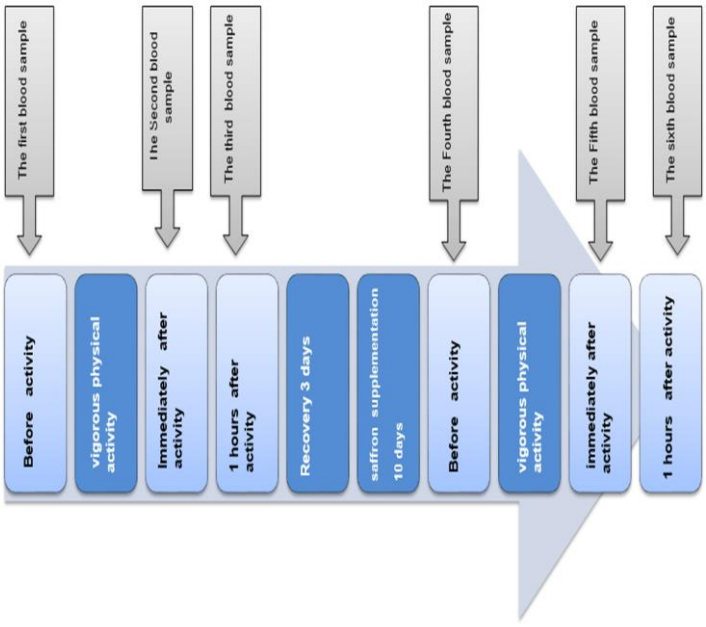

Fig. 1. Research scheme

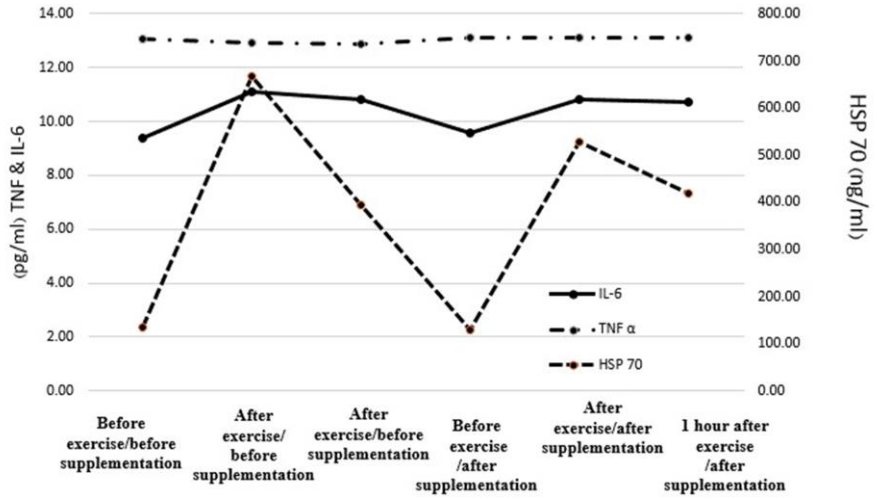

Fig. 2. Changing trend of variables TNFα, IL-6 and HSP70 in six measurement phases

Study design and laboratory investigation

Figure 1 shows the research scheme, sampling frequency and times of supplementation. Containing 300 mg of dried stigmas of the *Crocus Sativus* capsules, supplements were administered once a day. Blood samples were collected from subjects prior to, immediately and one hour after the strenuous physical activity to measure HSP70, IL-6 and TNFα. 3 days after recovery, the subjects were given 300 mg of dried stigmas of *Crocus Sativus* in capsules once a day for ten days [18]. Subsequently, blood samples were obtained from the subjects again prior to, immediately and one hour after one section strenuous physical activity. The serums were isolated from the elbow of the right hand. Then, the sterile blood was poured into labeled anticoagulant-free test tubes and incubated for 60 minutes at room temperature. Then, they were centrifuged at 2000 RPM for 20 minutes. The supernatant transparent solution (serum) was carefully isolated through Sampler 1000 in a way not to mix with red blood cells. The serum was kept at -80 °C until testing.

Preparation of Crocus Sativus capsules

Crocus Sativus stigmas were supplied from Ghaen by Novin Saffron Co. (Iran). Each capsule containing 300 mg of dried *Crocus Sativus* stigmas was formulated by a digital scale with precision of 0.0001.

Analysis of Crocus Sativus

The results of physical and chemical tests on *Crocus Sativus* at Novin Saffron Co. have been described in the Table 2**.** In this regard, 100 grams of *Crocus Sativus* were assessed according to Iranian national standards.

In accordance with Iranian national standards 259-1, 259-2, physicochemical properties of

Table 2. Analysis of *Crocus Sativus*

The maximum style with stigma (mass %)	0.23
External substances (mass %)	0
The maximum external plant substances and to environment external substances	
Maximum humidity and volatile matter (mass %)	6.52
Maximum total ash based on dry matter (mass %)	5.01
Maximum ash insoluble in Hydrochloric acid (mass %)	0.23
Soluble extract in cold water based on dry matter (mass %)	59.71
Minimum Picrocrocin per dry matter (Maximum absorption at 257 nm)	88.23
Safranal per dry matter (Maximum absorption at 330 nm)	35.19
Minimum crocin per dry matter (Maximum absorption at 440 nm)	236.95
Net weight	100

Table 3. Serum concentration of inflammatory biomarkers in different phases of physical activity following supplementation with *Crocus Sativus*

		TNF_{α} (pg/ml)	IL-6 (pg/ml)	HSP 70 (ng/ml)
Before supplementation	Before activity	13.04 ±1.12	9.38 ±0.49	131.92±22.56
	Immediately after activity	12.90±1.03	11.08±1.48†	680.25±127.86‖†
	1 hour after activity	12.88 ±1.46	10.81±1.51†	389.17±99.48†‡
After supplementation	Before activity	13.10 ±1.24	9.59±0.51	130.00±45.92
	Immediately after activity	13.12 ± 1.34	10.83±1.14*	528.18±111.63*
	1 hour after activity	13.10 ± 1.52	10.74±1.01*	415.42±189.41*¶

† significant difference to values of before strenuous physical activity and before supplementation (P<0.05), * significant difference to values of before strenuous physical activity and after supplementation (P<0.05), ‖ significant difference to values of immediately after strenuous physical activity and after supplementation (P<0.05), ‡ significant difference to values of immediately after strenuous physical activity and before supplementation (P<0.05), and ¶ significant difference to values of immediately after strenuous physical activity and after supplementation (P<0.05).

saffron using a spectrophotometer model JENWAY 6305 uv/vis were measured.

Ethical considerations

The study protocol was evaluated and accepted by ethical committee of Science and Research Branch, Islamic Azad University, Tehran, Iran. All participant received adequate information about the study protocol and possible good and ill effect of protocol. They entered the study deliberately and were free to quit the protocol upon their request.

Statistical analysis

All data was analyzed using arithmetic mean and standard deviation. Since Kolmogorov–Smirnov Bonferroni showed no significant results, analysis of variance with repeated measurements and Bonferroni follow-up tests were performed. All statistical calculations were performed with the SPSS (Statistical Package for Social Sciences) for Windows 15.0 software (SPSS, Chicago, IL). The level of statistical significance was pre-set at $p< 0.05$.

Results

Results of changes in inflammatory biomarkers following different physical activity and intervention are shown Figure 2 and Table 3**.** A session strenuous physical activity without *Crocus Sativus* supplementation significantly increased levels of IL-6 and HSP70 at the immediately and after one-hour physical activity while leaving no effect on TNFα level at the before, immediately and after one-hour physical activity.

Moreover, a session strenuous physical activity with *Crocus Sativus* supplementation could significantly reduce the levels of HSP70 immediately after the workout. It also reduced the IL-6 levels, although it was not statistically significant. Finally, it left no effect on TNFα levels.

Figure 2 displays the changing trend of IL-6, HSP70 and TNFα at six measurement phases.

Table 3 displays descriptive statistics and summary of results obtained by analysis of variance with repeated measures concerning the variables of IL-6, TNFα and HSP70 in six measurement phases.

Discussion

The principal components of *Crocus Sativus* are crocin, crocetin and safranal. These substances play a key role in the plant's pharmacological activities and antioxidant functions found in the reddish-orange stigma of the *Crocus Sativus* flowers. The *Crocus Sativus* compounds with pharmacological effects are bitter substances derived from safranal and crocetin carotenoid pigments. A notable example of bitter substances is Picrocrocin [12]. In a study on the anti-inflammatory effect of *Crocus Sativus*, it was indicated that *Crocus Sativus* can inhibit cyclooxygenase activity by flavonoids, tannins, anthocyanins, alkaloids and saponins [19].

In this study containing 300 mg of dried stigmas of the *Crocus Sativus* capsules, supplements were administered once a day. According to the analysis of *Crocus Sativus* the Effective ingredients, (crocin, Picrocrocin and safranal) each capsule respectively (Maximum absorption at 440 nm is 0.711, maximum absorption at 257 nm is 0.265 and maximum absorption at 330 nm is 0.1.

In research conducted by Mohamadpour

(2013) analysis of *Crocus Sativus* the Effective ingredients, (crocin, Picrocrocin and safranal) respectively showed (Maximum absorption at 440 nm is 225.8, maximum absorption at 257 nm is 83.83 and maximum absorption at 330 nm is 33.73 (*Crocus Sativus* samples were taken from Khorasan, Ghaen)[20].

It is expected to increase the amount of active ingredients in each capsule by increasing the dried stigmas of the *Crocus Sativus* or by increasing the amount of enrichment can increase the effectiveness of *Crocus Sativus*.

Furthermore, it has been found that the anti-inflammatory properties of *Crocus Sativus* are based on its antioxidant activity. Crocetin may decrease the level of nitric oxide and MDA, which in turn inhibits the reactive oxygen and nitrogen species, resulting in lower oxidative stress. Additionally, it reduces the response expression of pro-inflammatory cytokines TH1, which leads to suppression of nitric oxide synthase iNOS and weakening of the mobilization of neutrophils. Subsequently, decreased lipid peroxidation and lower nf-kb will curtail inflammation [21]. In research conducted on antioxidant properties of *Crocus Sativus*, it was shown that crocin significantly increases the gene expression of catalase and superoxide dismutase, thus curtailing the oxidative stress [22]. Some researchers believe that taking antioxidants can reduce free radicals. As a result, it delays or stops the inflammatory reactions and cell damage process. Owing to its antioxidant effects due to the presence of crocin, safranal, carotenoids, flavonoids as well as crocin and crocetin, *Crocus Sativus* tends to have greater antioxidant properties than other compounds[23].

Moreover, TNFα is a primary mediator of local inflammatory processes as well as initiator of systemic acute phase response [10]. However, the TNF-α composition can be a strong stimulant for production of IL-6. The general effect of IL-6 along with TNF-α can induce acute phase proteins and fever. The local function of cytokines could be detrimental and in the absence of control, can spread infection and cause shock. Moreover, the TNF-α composition is known as a metabolic cytokine, decreasing protein synthesis in the muscles and escalating their decomposition[11].

The TNFα levels in male climbers in two measurement phases including pre- strenuous physical activity and after were not significantly different from the values of after strenuous physical activity and before *Crocus Sativus* supplementation. The absence of strenuous physical activity effect on serum levels of TNFα could be associated with the duration of strenuous physical activity lasting 45 minutes, since most of the previous research observing the TNFα-inducing changes after strenuous physical activity protocol had administered longer strenuous physical activity [13]. These results were consistent with those obtained by Ostrowski et al. (1998) [24], Isanejad et al. (2015) [25] and Suzuki et al. (2006) [26]. Conversely, the results were inconsistent with those obtained by Cooper et al. (2005) [27], Ziemann et al. (2012)[28], Ziemann et al. (2013) and Riemann et al. (2013). This could be due to the duration of activity on Riemann et al. in 2013 (marathon) which was longer than that in the current study. Meanwhile, Ziemann et al. (2013) examined the variations of TNFα after the competition season and 2 weeks later.

Moreover, the results this study showed that IL-6 levels in male climbers in two measurement phases, including before the activity and before supplementation were significantly different from the values of after activity and before *Crocus Sativus* supplementation. Additionally, IL-6 levels significantly increased after strenuous physical activity. Furthermore, the IL-6 levels in male climbers in three measurements were significant different. In fact, IL-6 increased after strenuous physical activity, remained stable at a high level one hour after the end of the activity and did not return to baseline levels.

IL-6 is known as the primary cytokines involved in the response to exercise and inflammation appearing in blood and increasing clearly in the plasma[29]. It depends on intensity, duration, muscle mass involved in sports and physical fitness level of each individual [30]. The mechanism is probably related to the fact that strenuous exercise releases pro-inflammatory cytokines, which in turn produce other anti-inflammatory cytokines such as IL-2, IL-6 and IL-10. It seems that short-term or moderate intensity training will not significantly alter IL-6 levels [31]. Based on some studies, however, strenuous eccentric resistance exercises that focus on large masses of muscle (even in a single training session) have shown a significant increase in levels of IL-6 [32]. Therefore, this study expected an increase in IL-6 after strenuous activity given the exercise protocol involving large muscles (quadriceps) and the occurrence of eccentric training.This was

consistent with the research conducted by Ostrowski et al. (1998), Zaldivar et al. (2005)[27] Glysen (2007)[33], Nieman (2005)[34], Riemann et al. (2013) and Suzuki et al. (2006), whereas it was inconsistent with the research by Isanejad et al. (2012).

Earlier research suggested that the emergence of IL-6 in blood circulation is associated with muscle damage. However, new research clearly shows that even without any muscle contraction there will be a significant increase in IL-6 plasma levels which depends on the duration and types of sports activities as well a sympathetic adrenal-dependent response. Moreover, it has been proven that repeated penetration of neutrophils and macrophages into damaged muscle tissue occurs during 6 to 48 hours after exercise, where the activated macrophages will release IL-6 as part of the inflammatory response [35]. Some scholars believe that protein fragments released from damaged muscles collide with white blood cells and other cells (such as fibroblasts), thus releasing cytokines, while others have asserted that the increased body temperature releases catecholamines, which in turn activate the immune cells indirectly involved in the release of cytokines during and after sports activity [36].

The results of some studies suggested that exercise can similar to other stimulants lead to metabolic changes and the production of HSP70. In fact, the level of heat shock protein in various organs of the body increases after long workouts [37, 38]. Nevertheless, some studies reported no change. One of the common points in the results obtained by various studies is the impact of intensity and duration of strenuous physical activity. For instance, a study found that increased intensity of exercise can enhance the level of this protein. The higher the intensity, the greater the production of HSP70 [37]. In another study, it was shown that by increasing the workout distance and duration, the level of HSP70 increases. Long-term exercise leads to oxidative stress which in turn stimulates the production of heat shock proteins as an important part of the cellular protective response against damage.

An eccentric exercise session increased the levels of mRNA and HSP70 over days after exercise, leading to the response of heat shock protein to changes in muscle function. Moreover, the expression of HSP70 is stimulated by cytokines. Metabolic disorders (e.g. low pH), stress or increased expression of HSP70 are essential in curtailing temperature. Extreme mechanical stress, muscle damage and inflammation are the primary stimulants for greater expression of heat shock protein within the exercise protocol [39]. The results of this study showed that the level of HSP70 among male climbers in two measurement phases including pre-exercise and after supplementation were significantly difference from the values of after activity and before *Crocus Sativus* supplementation. In fact, the HSP70 levels after the activity and before supplementation were significantly greater than those before the activity. Furthermore, the HSP70 slightly increased after strenuous activity but then continued to rise for one hour after the end of activity and never returned to the baseline. The HSP70 levels after an hour of activity and before *Crocus Sativus* supplementation significantly increased compared to the values of pre-exercise and post workout supplementation. One hour after, the HSP70 values reduced significantly compared to the values of immediately after the exercise, even though it still did not return to the resting levels. This was consistent with the research conducted by Pantchart et al. (1991), Peak et al. (2005), Michelson et al. (2013)[40], Isanejad et al. (2012), Touchberry, et al. (2012)[41] and Samelman et al. (2000)[42].

Limitation of the study

This study was limited to the small number of subjects and also short time supplementation, and similar research with more subjects and longtime supplementation to find out the effectiveness of *Crocus Sativus* on il6, tnfα and hsp70.

Conclusions

The results indicated that *Crocus Sativus* owing to its anti-inflammatory and antioxidant properties can leave protective effects on HSP 70 responses after strenuous physical activity.

Conflict of Interests

The authors declared no conflicts of interests.

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